ASPHALT INSTITUTE
Executive Offices and Research Center
Research Park Drive
P.O. Box 14052
Lexington, KY 40512-4052
USA
Telephone 859-288-4960
FAX No. 859-288-4999

BIO-DIRECTED FRACTIONATION OF LABORATORY-GENERATED ASPHALT FUMES: RELATIONSHIP BETWEEN COMPOSITION AND CARCINOGENICITY

ANTHONY J. KRIECH[1] JOSEPH T. KUREK[1], LINDA V. OSBORN[1], GARY R. BLACKBURN[2], AND FRED M. FEHSENFELD1

[1]Heritage Research Group, 7901 West Morris Street, Indianapolis, IN 46231, USA
[2]Petrolabs Inc., 133 Industrial Drive, Ivyland, PA, 18974, USA

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ABSTRACT

In the 1980's the National Institute of Occupational Safety and Health (NIOSH) [1,2] sponsored mouse skin-painting bioassays of roofing asphalt fume condensates generated at a temperature of 316ºC using a laboratory apparatus. Also included in the study were five HPLC fractions (designated A-E), which were skin-painted individually and in various combinations. The whole fume condensates and two of the five fractions (B and C) were shown to be carcinogenic. The present study repeated the fume generation and fractionation phases of the NIOSH study using the same asphalt, fume generation apparatus, and HPLC method. The carcinogenic fractions, B and C, were then subfractionated by cyanopropyl and silica gel column chromatography. Biological activity was tracked using the Modified Ames Assay, while relative PAC content was monitored using a newly developed fluorescence method. The results were consistent with the

hypothesis that relatively high molecular weight PACs were largely responsible for the carcinogenic activity of the NIOSH fumes.

Key Words: Asphalt, Fumes, Mutagenicity, Carcinogenicity, Polycyclic Aromatic Compounds, Fluorescence

INTRODUCTION

In the past twenty years, numerous studies have addressed the question of asphalt fume carcinogenicity. The principal objective of these studies has been the determination of whether workers in the asphalt industry incur significant carcinogenic risk from exposure to asphalt fumes.

While this work has contributed substantially to our understanding of asphalt fume chemistry and toxicology, it has fallen short of meeting the standards necessary for use in hazard assessment or risk estimation. The principal problems have been the chemical dissimilarity of the fumes to normal workplace fumes and/or the lack of relevance of the measured endpoints to the question of worker risk.

Perhaps the most comprehensive and conclusive studies of asphalt fume carcinogenicity are two NIOSH-sponsored mouse skin-painting assays of laboratory-generated fume condensates [1,2]. These studies unequivocally demonstrated that the fume condensate itself and two of five High Performance Liquid Chromatography (HPLC) fractions derived from it (B and C) were carcinogenic, while the remaining three fractions (A, D and E) were not.

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Because the mode of fume generation in these studies was designed to maximize yield rather than mimic field fume composition, and hence toxicology, the relevance of these findings to the central question of workplace hazard has been seriously questioned. But despite this drawback, the materials from this study -- as the only true asphalt fume samples tested in a rigorous carcinogenicity bioassay -- represent an invaluable research tool.

The purpose of the present study was to further characterize the compounds responsible for the carcinogenicity of the NIOSH fumes, and to assess the performance of two methods -- one chemical and one biological -- for tracking these compounds during fractionation. Ultimately, better characterization of the carcinogenic species would permit a determination of whether they are present in workplace asphalt fumes.

NIOSH generously supplied retains of the original asphalt, as well as the fumes and fume fractions for the present study. Because the retains were available in very limited supply, new fume samples were also generated from the asphalt and fractionated by HPLC, using equipment closely similar to that employed in the original studies. The new preparations were then subfractionated by a combination of cyanopropyl and silica gel column chromatography.

The Modified Ames mutagenicity assay [3], shown to have a high correlation with carcinogenic potential of petroleum derived materials in animal skin painting studies [4] was used to monitor biological activity. In addition, a newly developed fluorescence method was used to track the distribution of Polynuclear Aromatic Compounds (PACs) during the fractionation.
The results of the study are consistent with the conclusion that a high proportion of the carcinogenic activity in NIOSH fumes (perhaps all) was mediated by PACs (both hydrocarbon and heterocycle, alkylated and unalkylated) in the 4-6 annulated ring class. The study also

demonstrated that both the biological and the chemical monitoring methods accurately predicted the carcinogenic potency of the various fractions and combinations of fractions, as determined in the skin-painting bioassay.

MATERIALS AND METHODS

Laboratory Fume Generation

Laboratory fume generation was conducted as described by Sivak et al. [2].

Mouse Skin-painting bioassays

Mouse skin-painting bioassays were conducted as described by Sivak et al. [2].

Modified Ames Test

The Modified Ames Test was performed in accordance with the procedures described in ASTM Standard Method E 1687-95 [3]. In some cases, the quantity of sample available was too small to permit testing at the full number of doses specified.

HPLC fractionation of Fume Condensate

Fractionations were conducted as described by Sivak et al. [2], except that a lower flow rate (150 ml/min) was employed.

Cyanopropyl/Silica Gel Subfractionation of Fume Fractions

Waters Sep-Pak Vac 12cc (2g) CN Cartridges were used for the cyanopropyl separations. Cartridges were pre-rinsed with methylene chloride (MeCl2) and hexane. Approximately 10 milligrams of fume or fume fraction were loaded onto a cartridge in hexane and eluted according to Figure 1, using 35 ml hexane, 35 ml $MeCl_2$, and 30 ml of $MeCl_2$/MeOH.

The hexane fraction was then reduced to 3-5 ml and loaded onto a 2 g column of baked silica gel capped with 3 mm of baked Na_2SO_4. After discarding the first 10 ml of hexane, chromatography was conducted according to Figure 1 using about 30 ml of each solvent.

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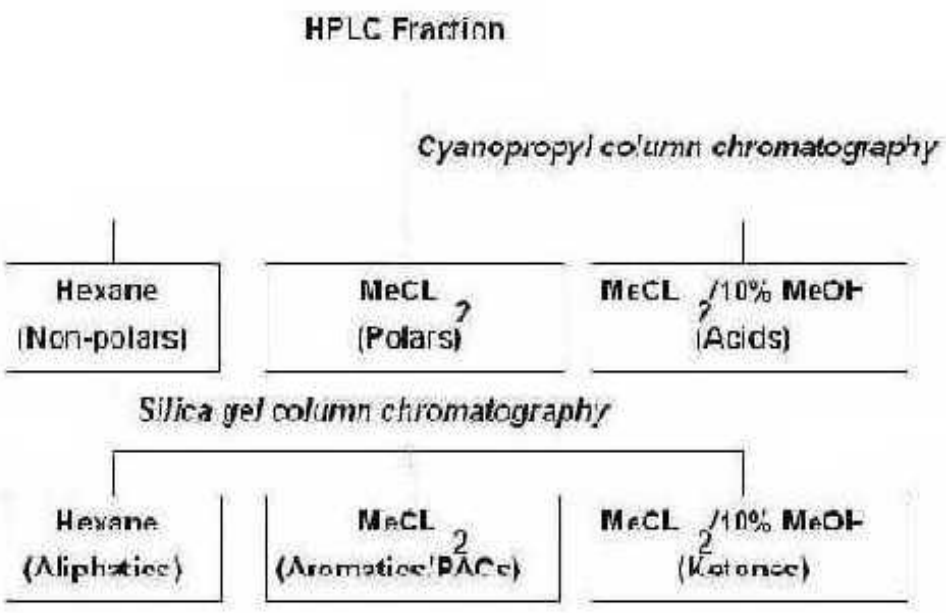

Figure 1. Cyanopropyl / Silica Gel subfractionation of asphalt fume fractions

Fluorescence Assay

Fumes or fume fractions (£ 25 mg) were dissolved in cyclohexane and brought to a final volume of 1.0 ml. The resulting stock solution was diluted as necessary. Final volumes were adjusted to

10 ml. A preliminary reading of fluorescence intensity was then taken using a Perkin Elmer LS50B Luminescence Spectrometer. Starting emission range was 410 to 420 nm, with excitation at 385 nm. Both slits were set at 3 nm. Scan speed was 1000 nm/min. Emission intensities were read at 415 nm. Samples were diluted to give intensities within the linear range of the instrument. Cyanopropyl cleanup of the entire 10 ml sample was then performed. Emission intensities were multiplied by the appropriate dilution factors, divided by the weight in grams, and then divided by 10,000 for convenience and expressed as Emission Units/gram (EU/g)

RESULTS

Comparison of Yield and Mutagenicity Index of New Fume and Fume Fraction Preparations with Those Evaluated in the Original NIOSH Studies

Table 1 provides a comparison of the yield and Mutagenicity Index of each of the five fractions prepared from the original NIOSH 316ºC fume condensate (columns 0) with those from two subsequent preparations of the analogous fractions (columns 1 and 2). The data indicate that the reproducibility of the fume generation and fractionation was quite good, although somewhat larger yields of Fraction A and correspondingly smaller yields of Fraction B were observed in the two later fractionations.

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TABLE 1. Yields, MIs, and CIs of Whole Fume and HPLC Fractions A-E.

	Weight Percent			Mutagenicity Index (MI)			Carcinogenicity Index (CI)[1]
Phase[2]	**0**	**1**	**2**	**0**	**1**	**2**	**0**
Sample							
Whole Fume	100	100	100	4.4	4.1	4.1	9.0
Fraction A	65.5	74.6	69.4	3.6	3.0	1.5	0
Fraction B	8.1	3.4	5.0	29	28	30	3.4
Fraction C	10.6	6.8	11.5	25	20	19	6.6
Fraction D	11.4	11.8	14.1[3]	0	0	---	0
Fraction E	4.4	3.4		0	0	---	0

[1] CI = [percent of mice with tumor(s) / mean time (weeks) to first tumor] x 10
[2] Phase 0 = Original NIOSH Samples; 1 = 1st Heritage Fume Generation; 2 = 2nd Heritage Fume Generation
[3] Fractions D and E were combined in Phase 2 Heritage Fume Generation and Separation

The Mutagenicity Indices (MI) of Fractions B and C -- those with carcinogenic activity -- remained relatively constant, while the MI of Fraction A was lower for the later preparations despite the higher mass yields. Since the HPLC pump rate used in preparations 1 and 2 was lower than that used in the original NIOSH separation, these results may reflect somewhat better separations and therefore less "spillover" of mutagenic species from Fraction B into A. Nonetheless, even with the higher content of B-type material in the original Fraction A, none of the skin-painting groups treated with A developed tumors (C.I. in Table 1).

Correlation of Mutagenicity with Carcinogenicity for Whole Fume and Fractions A - E

Figure 2 shows the correlation between mutagenicity (MI) and carcinogenicity, expressed as Carcinogenicity Index per g (CI/g = [% mice with tumor(s) ÷ time in weeks to 1st tumor x 10] per gram of fraction skin-painted). The index is multiplied by ten simply to give convenient values. CI/g is chosen because the animals were dosed in relation to the amount of fume each fraction represented of the whole fume thus normalizing the dose variable.

In addition to the five fractions and whole fume, four combinations of fractions as used in the NIOSH Study are also included in the correlation to provide additional non-zero data points. The R^2 (0.97) indicates that the Modified Ames Test is measuring an endpoint directly related to the carcinogenicity of the fractions. Other correlative studies [4,5] have demonstrated that the endpoint is PAC-mediated mutagenicity. Based on these results, the Modified Ames Test was considered an effective means of tracking the distribution of carcinogenic activity during the initial subfractionation studies.

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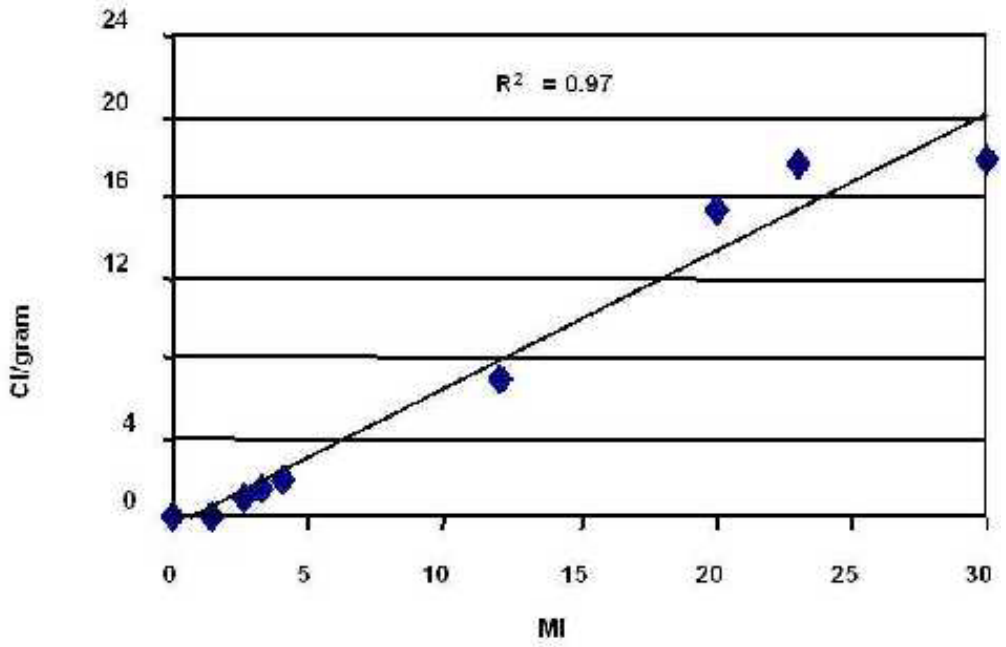

Figure 2. *Correlation between MI and CI/gram for fumes and fume fractions*

Subfractionation of Fractions A, B and C Using Cyanopropyl and Silica Gel Chromatography

Ten mg portions of each of the NIOSH fractions or combinations of fractions shown in Figure 2 were subjected to the fractionation scheme shown in Figure 1. Because of the small amount of material available for separation, biological activity could not be tracked using the Modified Ames Test, and mass recoveries could not be calculated.

Sample	MI	CI/g
Whole Fume (1)	4.1	1.73
A (2)	1.5	0
B (2)	30	17.8
C (2)	20	15.3
D (1)	0	0
E (1)	0	0
A + B (2)	2.6	0.97
A + C (2)	3.3	1.35
B + C (2)	23	17.5
B + C + D (1)	12	6.92

However, based on the knowledge that higher molecular weight PACs are the principal carcinogens/mutagens in the NIOSH fume condensate and that these compounds are highly fluorescent, a fluorimetric assay was developed as a means of tracking the efficiency of fractionation. The fluorescence excitation and emission wavelengths (385 and 415 nm) were chosen so as to discriminate between the 4-6 ring compounds of interest and the 1, 2 and 3-ring aromatics, thereby making the cyanopropyl/silica gel separation unnecessary. The approach, optically isolating the components of interest, yielded a good correlation with CI/g for n =15 (R^2 = 0.89). However NIOSH Fraction D was a false positive under these conditions, because of the fluorescence of noncarcinogenic polar compounds. Therefore, a short cyanopropyl cleanup step was added to remove the polar materials. The additional step improved the correlation to an R^2 of 0.94.

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When an additional twenty-five samples (some of them from the later fume generation and HPLC fractionations) were subjected to cyanopropyl cleanup followed by fluorescence measurement, the results shown in Figure 3 were obtained. The strong correlation between fluorescence and biological activity is maintained (R^2 = 0.95), confirming the value of the fluorescence method as a detector of PACs with carcinogenic potential.

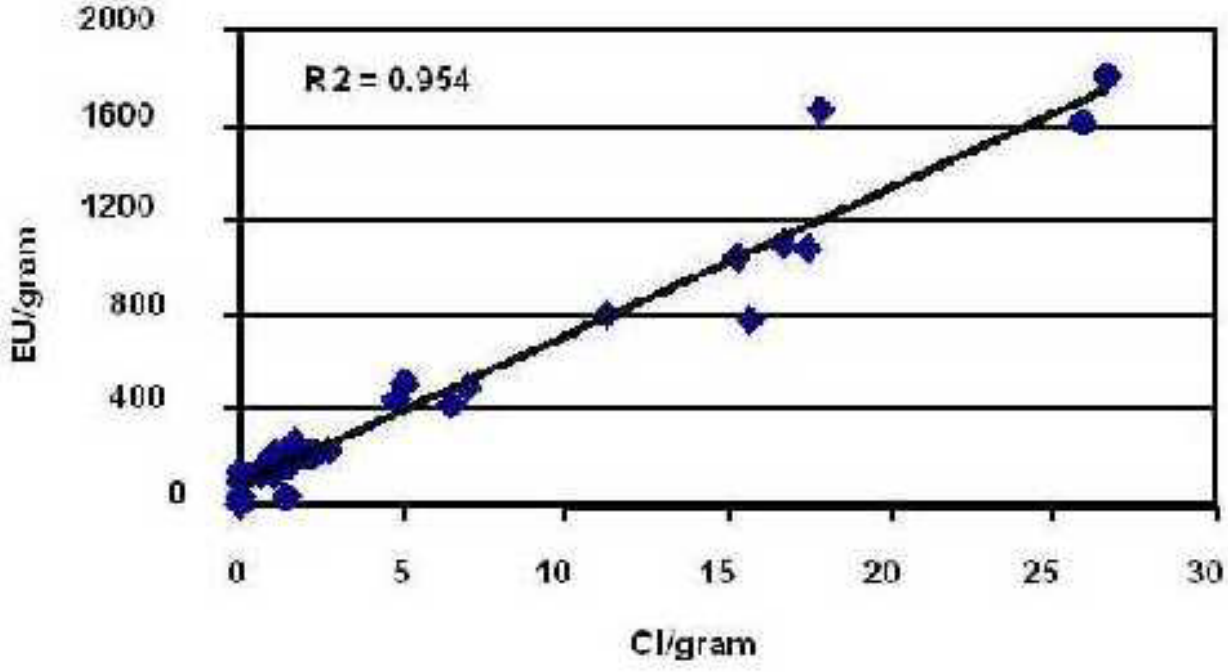

FIGURE 3. Correlation between CI/Gram and EU/Gram for 40 fumes and fume fraction

Sample	CI/gram	EU/gram	Sample	CI/gram	EU/gram
A (2)	0	107	Whole (0)	1.73	214
A (1)	0	134	ABCD (1)	2.1	209
D (1)	0	15	ABCD (0)	2.1	221
E (1)	0	7.0	ABCDE (1)	2.24	207
A (0)	0	87	ABCDE (0)	2.29	211
D (0)	0	44	ABCE (0)	2.71	230
E (0)	0	7.6	ABCE (1)	2.72	232
ABDE (1)	0.66	162	BCDE (0)	4.8	442
ABDE (0)	0.69	125	BCD (0)	5.1	508
AB (1)	0.92	188	BCDE (1)	6.52	424
AB (2)	0.97	220	BCD (1)	6.92	496
AB (0)	0.98	129	B (0)	11.2	798
AC (1)	1.34	202	C (2)	15.3	1041
AC (2)	1.35	211	BC (0)	15.6	771
AC (0)	1.44	202	C (0)	16.7	1100
ACDE (1)	1.46	29	BC (2)	17.5	1077
ACDE (0)	1.54	170	BC (2)	17.5	1082
Whole	1.73	217	B (2)	17.8	1656
Whole	1.73	266	C (1)	25.9	1615
Whole	1.73	215	B (1)	26.7	1800

(0) = Original NIOSH Skin Painting Fume
(1) = Regenerated by Heritage (6-1994)
(2) = Regenerated by Heritage (12-1994)

Since the more highly condensed PACs have emission maxima at longer wavelengths than the 2 and 3 ring aromatics and with the known role of 4-6 ring PACs in the carcinogenicity of petroleum materials [4,5], this high correlation between EU/g and CI/g is not surprising.

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DISCUSSION

This study has demonstrated that the NIOSH-sponsored laboratory fume generation and fractionation methods are highly reproducible, and that fumes and fume fractions produced using the methods are representative of those assayed in the original skin-painting bioassay. Since the actual skin-painting solutions are now largely depleted, the ability to produce new samples -- particularly of the active fractions -- makes possible the further elucidation of the chemical species responsible for the carcinogenicity observed.

The subfractionation work described in this report is consistent with the conclusion that those species are PACs with four to six annulated rings. That premise is based on the following observations:

1. MI, as measured in the Modified Ames Test, correlated well with carcinogenic potency for whole fume, the five fractions, and several combinations of fractions, i.e. the carcinogenic

Fractions B and C had high MIs while the noncarcinogenic A, D and E had 0 or low MIs. In combinations of noncarcinogenic with carcinogenic, MI again reflected the content of B- and/or C-derived material. Other work [4,5] has shown that MI strongly correlates with an oil's content of 3-7 ring PACs, and that, conversely, oils containing primarily one-, two-, and three-ring aromatics are, at most, weakly mutagenic.

2. The relatively high fluorescence intensities of Fractions B and C at 415 nm are consistent with the presence of 4-6 ring PACs whereas the low fluorescence of Fraction A, which fluoresced strongly at shorter wavelengths, is consistent with the presence of 2 and 3-ring PACs [6].
3. The subfractionation method evaluated in this study segregated mutagenic activity (and fluorescence activity at 385ex/415em) to the cyanopropyl/silica gel $MeCl_2$ fraction. Little or no activity (nor fluorescence) was found in the other subfractions comprised primarily of other chemical types such as polars, acids, aliphatics or ketones [2].

These results indicate that 4-6 ring PACs played a significant role in the dermal carcinogenicity seen in the NIOSH animal studies of laboratory fumes, and that both the Modified Ames Test and the fluorescence endpoint developed here are reliable methods of detecting the presence of such compounds. As a result, both assays have been shown to be valuable research tools in the ongoing effort to identify the specific compounds or classes of compounds that are responsible for the dermal carcinogenicity of the NIOSH laboratory fumes in animals.

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The results also suggest that the Modified Ames Test and the fluorescence method described here may, with additional research and validation, prove to be effective screening tools as part of an overall valuation of the carcinogenic hazard of workplace asphalt fumes. The most discriminating method in this study for achieving this goal is the Modified Ames Test, which can selectively measure the effects of carcinogenic PACs in the presence of a preponderance of noncarcinogenic analogs. However, the Modified Ames Test has the shortcoming that reasonably large amounts of fume are required for testing (generally 200 mg or more). Since it is difficult, if not impossible, to obtain quantities of fume this large in actual workplace operations, more sensitive screening methods, which can in turn be correlated with biological endpoints such as MI, will be needed for this effort. The present study has shown that the fluorescence endpoint developed here has the sensitivity required for field fume studies. Moreover, the endpoint is capable of distinguishing between samples containing primarily low molecular weight aromatics and those enriched in the 4-6 ring compounds of biological interest. And, finally, the fluorescence endpoint developed in this study correlates strongly with MI and CI for both the carcinogenic and noncarcinogenic fractions evaluated in the NIOSH skin-painting bioassay. In light of the very high compositional variability of asphalt and asphalt fumes, additional research is needed to determine whether the correlations found in this work extend to other types of asphalt or to asphalt fumes generated under field conditions.

ACKNOWLEDGMENTS

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